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Markedly Reduced Bile Acid Synthesis but Maintained Levels of Cholesterol and Vitamin D Metabolites in Mice with Disrupted Sterol 27-Hydroxylase Gene*

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Sterol 27-hydroxylase is important for the degradation of the steroid side chain in conversion of cholesterol into bile acids and has been ascribed a regulatory role in cholesterol homeostasis. Its deficiency causes the autosomal recessive disease cerebrotendinous xanthomatosis (CTX), characterized by progressive dementia, xanthomatosis, and accelerated atherosclerosis.

Mice with a disrupted *cyp27* (*cyp27*$^{-/-}$) had normal plasma levels of cholesterol, retinol, tocopherol, and 1,25-dihydroxyvitamin D. Excretion of fecal bile acids was decreased (<20% of normal), and formation of bile acids from tritium-labeled 7α-hydroxycholesterol was less than 15% of normal. Compensatory up-regulation of hepatic cholesterol 7α-hydroxylase and hydroxymethylglutaryl-CoA reductase (9- and 2–3-fold increases in mRNA levels, respectively) was found. No CTX-related pathological abnormalities were observed. In CTX, there is an increased formation of 25-hydroxylated bile alcohols and cholestanol. In bile and feces of the *cyp27*$^{-/-}$ mice only traces of bile alcohols were found, and there was no cholestanol accumulation.

It is evident that sterol 27-hydroxylase is more important for bile acid synthesis in mice than in humans. The results do not support the contention that 27-hydroxylated steroids are critical for maintenance of cholesterol homeostasis or levels of vitamin D metabolites in the circulation.

Sterol 27-hydroxylase is a mitochondrial species of cytochrome P-450 with a broad tissue and organ distribution and with a broad substrate specificity (for a review, see Ref. 1). The enzyme is important for bile acid biosynthesis but has also been ascribed a role in connection with cholesterol removal from extrahepatic tissues, in regulation of cholesterol homeostasis, and in metabolism of vitamin D.

Sterol 27-hydroxylase is responsible for the first step in the degradation of the steroid side chain in connection with bile acid biosynthesis in the liver. In the major pathway to bile acids in mammalian liver, 7α-hydroxylation of cholesterol is the first and rate-limiting step, and 27-hydroxylation occurs at a later stage with a 7α-hydroxylated intermediate as substrate. In an alternative pathway, 27-hydroxylation of cholesterol is the first step in the sequence, followed by a 7α-hydroxylation catalyzed by a specific oxysterol 7α-hydroxylase (1). In a recently discovered minor pathway, extrahepatic sterol 27-hydroxylase converts cholesterol into 27-hydroxycholesterol or 3β-hydroxy-5-cholestenoic acid (2, 3). The latter compounds are transported to the liver and converted into bile acids.

Sterol 27-hydroxylase has also 25-hydroxylase activity toward vitamin D (4) and 1α-hydroxylase activity toward 25-hydroxyvitamin D (5). Since there is also a microsomal cytochrome P-450 that catalyzes 25-hydroxylation of vitamin D (6), the relative importance of the sterol 27-hydroxylase is not known.

27-Hydroxycholesterol, formed from cholesterol by the sterol 27-hydroxylase, is a potent down-regulator of cholesterol synthesis in cultured cells (for reviews see Refs. 7 and 8). On the basis of this, and on the basis of studies with sterol 27-hydroxylase inhibitors, sterol 27-hydroxylase has been suggested to have a regulatory role in cholesterol homeostasis. The relative importance of this mechanism is controversial, however. Based on studies with cholesterol specifically deuterium-labeled in the 27-position, which retards the rate of 27-hydroxylation, we showed that sterol 27-hydroxylase activity is of little or no direct importance for cholesterol-induced suppression of cholesterol synthesis in mouse liver (9).

It is now well documented that patients with the rare disease cerebrotendinous xanthomatosis (CTX)[1] have a deficiency of sterol 27-hydroxylase (for a review, see Ref. 10). Recently, a number of mutations have been defined in the sterol 27-hydroxylase gene of these patients (11–20). As a result of the enzymatic defect, patients with CTX have a reduced synthesis of bile acids, in particular chenodeoxycholic acid (10). Cholic acid is formed in these patients by a pathway involving 25-hydroxylated bile alcohols as intermediates. Under normal conditions this alternative pathway is of little or no importance, both in rats and man (21, 22). Due to the reduced formation of bile acids, the negative feedback of the cholesterol 7α-hydroxylase is reduced, resulting in an up-regulation of this enzyme. As a

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[1] The abbreviations used are: CTX, cerebrotendinous xanthomatosis; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; HMG-CoA, hydroxymethylglutaryl-CoA.

consequence, gram amounts of bile alcohols are formed and excreted in bile and feces. Another consequence of the disease is excess formation and accumulation of cholestanol (10). Most of this formation seems to be secondary to the accumulation of an intermediate in bile acid biosynthesis, 7α-hydroxy-4-cholesten-3-one. This steroid can be converted into cholestanol by hepatic enzymes.

Patients with CTX have normal cholesterol levels in the circulation (17). Despite this they develop xanthomas and premature atherosclerosis. The possibility has been discussed that this may be due to the reduced elimination of cholesterol from macrophages by the sterol 27-hydroxylase (1–3).

Disturbances in vitamin D metabolism have been described in a few cases of CTX (23), but this does not seem to be a general finding.

Studies on patients with CTX have provided important information about the role of the sterol 27-hydroxylase in man. The importance of this enzyme for bile acid formation, cholesterol homeostasis, and vitamin D metabolism may, however, be different in different species. A lack of an enzyme may activate compensatory mechanisms that may be different in different species. In order to evaluate further the role of the sterol 27-hydroxylase in mammals, we have produced and characterized mice deficient of the enzyme.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse cyp27 and Construction of the Targeting Plasmid—Oligonucleotides complementary to the putative exon 3 of the rat *cyp27* were prepared (24–26). Oligonucleotides e (5′-AGGACAGCAGTGGTACCATCTGCG-3′) and f (5′-CTTCCAAGGCAAGGTGGTAAAGAAGA-3′) were used to amplify 197 bp containing the putative mouse exon 3 (Fig. 1). The PCR product was used to probe a mouse 129SV Genomic Library (Lambda Fix2, Stratagene, La Jolla, CA). A positive clone designated 24(1), which includes 13.1-kb mouse *cyp27* gene sequences, was isolated. A targeting construct was made to replace a 71-bp *Bam*HI fragment in exon 8 with a neo sequence (see Ref. 27, Fig. 1). To do so, a 7-kb *Bam*HI fragment, which includes sequences homologous to the human *CYP27* exons 2–8, was subcloned into a unique *Bam*HI site of the pPNT plasmid and designated pPNT-7. A 1.1-kb *Bam*HI/*Not*I fragment, which includes the 3′ end of the putative exon 8, exon 9, and the 3′-untranslated sequences, was purified from clone 24(1), subcloned into pSC301 plasmid, and designated pSC301-S. The *Bam*HI/*Sal*I 1.1-kb band of pSC301-S was then subcloned into the pPNT-7 plasmid that was previously digested by *Xho*I/*Not*I to make pCYP27T1. In the targeting construct the deletion of a 71-bp fragment from the putative mouse *cyp27* exon 8 is located upstream to the putative heme-binding site, highly conserved in human (28) and rabbit (29), and crucial for the activity of P-450 enzymes (30). For verification, the junctions between the *neo* and the *cyp27* sequences were sequenced and compared with the published *cyp27* rat cDNA (24).

Gene Targeting and Screening of the Homologous Recombinants—The targeting plasmid was linearized by cleavage with *Not*I. ES cell cultures and electroporations were performed as described (31). Ten to twelve days after electroporation, colonies resistant to 200 μg/ml G418 and to 2 μM ganciclovir (Syntex, Palo Alto, CA) were picked and passaged in clonal fashion. To screen the cells with correct targeting, a probe that identifies the 1.1-kb short arm was prepared by PCR using oligonucleotide a (5′-TGGTTCCCACAAACTCCCGGATCAT-3′), which is complementary to the putative rat 5′ exon 7 sequences, and oligonucleotide T3, which is complementary to the vector short arm sequence. Southern blot analysis resulted in identification of a 9- and a 12-kb *Xba*I fragment for the targeted and for the non-targeted alleles, respectively. To verify the planned disruption of the gene, a probe from the putative mouse exon 3 was prepared and used in Southern blotting to identify a 5- and a 16-kb *Xba*I fragment from the targeted and non-targeted alleles, respectively (Fig. 2*a*).

Generation of Germ Line Competent Chimeras and Mouse Breeding—Approximately 10 ES cells were injected into the blastocyte cavity of C57BL/6J embryos. Surviving blastocytes were transferred to the uteri of pseudo-pregnant CD-1 females. An average of two to three transfers were made per cell line. Chimeric animals were further bred to C57BL/6J animals to determine their germ line competency. F1 animals heterozygote for the *cyp27* disruption were crossed to produce mice homozygote to the knock-out *cyp27*.

PCR Genotyping of the F2 Siblings—To genotype the F2 siblings, a double PCR reaction was performed. PCR was carried out using oligonucleotide a (5′-TGGTTCCCACAAACTCCCGGATCAT-3′) mapped to the 5′ end of the putative exon 7, oligonucleotide b (5′-CCATAGCCAAAGGGCACAGAGCCAA-3′) mapped to the 3′ end of the putative exon 8, and oligonucleotide c (5′- ATCGCATCGAGCGAGCACGTACT-3′) complementary to *neo* sequences (Fig. 1). The PCR products of the targeted allele and the normal gene were 1- and 0.3-kb, respectively. To avoid the out competition of the 1-kb by the 0.3-kb PCR product, a triple amount of oligonucleotide a was used. The PCR conditions were as follows: denaturation for 5 min at 94 °C followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, and 75 °C for 1 min. To avoid PCR artifacts in genotyping, two additional, independent, reactions were carried out to identify the targeted allele and the normal allele. The oligonucleotides used for these reactions were c and d (5′-CCACCATGATATTCGGCAAGCAGG-3′, and a and b, respectively) (Fig. 3).

RNA Blot Analysis—For visualization of *cyp27* mRNA the following protocol was used. RNA was extracted from mice livers using Trizol reagent (Life Technologies, Inc.), quantified by spectrophotometry and visualized following electrophoresis on a 1.5% agarose gel stained by ethidium bromide. Twenty μg of total RNA were loaded on a 1.6% agarose gel, electrophoresed, and blotted as described (32). The blots were hybridized using a mouse *cyp27* exon 3 or a Syrian Hamster HMG-CoA reductase cDNA probe labeled by nick translation (Life Technologies, Inc.).

For visualization and quantification of *cyp7* mRNA the following protocol was used. Total cellular RNA was isolated with the Ultraspec™ RNA Isolation System (Biotecx Laboratories, Houston, TX). Electrophoresis of total RNA and poly(A)$^+$ RNA in agarose gels containing formaldehyde and blotting of the separated RNA onto nylon membranes (Hybond, Amersham Pharmacia Biotech, UK) was carried out by standard procedures (33). For the hybridization, cDNA probes for the rat *cyp7* and human actin were used, labeled with ^{32}P with the use of Pharmacia Oligolabeling kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was according to Gehring *et al.* (34). The blots were exposed to Fuji New x-ray films at −70 °C. Semi-quantitative analysis of the relative amount of mRNA was estimated by densitometry.

Immunoblot Analysis—Livers were homogenized in a lysis buffer containing 20 mmol of Tris-HCl, pH 7.7, 1 mmol of EDTA, and 0.25 M sucrose and centrifuged at 1000 × *g* for 5 min. The supernatant was precipitated at 5000 × *g* for 10 min and the mitochondrial fraction resuspended in the lysis buffer. The proteins were quantified using the Bio-Rad protein assay (Bio-Rad, Munchen, Germany). A total of 75 μg of protein was loaded on a 10% SDS-polyacrylamide gel and electrophoresed for 12 h at 15 mA. Electroblotting to cellulose nitrate (Schleicher & Schuell, Dassel, Germany) was carried out overnight at 20 V. Blocking of the membrane was carried out in a buffer containing 1.34 M NaCl, 0.03 M KCl, and 0.25 M Tris (TBS buffer) for 5 h at 4 °C. Incubation with primary polyclonal antibody directed against the mouse sterol 27-hydroxylase, extracted from rabbit serum (a gift from Dr. David W. Russell), was diluted 1/1000 in the blocking buffer (TBS) that also contained 0.05% Nonidet P-40 (Sigma). Incubation was carried out overnight at 4 °C. The filter was washed four times for 15 min each in a TBS buffer containing 0.1% SDS, 0.1% Nonidet P-40, and 0.5% deoxycholic acid sodium salt (Sigma-Aldrich Chemie, GmbH). Incubation with 1/2000 secondary antibody, anti-rabbit IgG horseradish peroxidase-linked whole antibody from donkey (Amersham Pharmacia Biotech, Buckinghamshire, UK) was carried-out for 2 h at room temperature. Development was carried out using the Amersham ECL detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). The film was exposed for 3 s and developed.

Pathological Analysis—Animals were maintained at the Hadassah-Hebrew University animal facility in a specific pathogen-free unit. Breeding pairs were set up to provide normal heterozygote and homozygote siblings of the targeted *cyp27*$^{-/-}$ mice. Weaning was carried out at the age of 3 weeks. The animals were genotyped and grouped. Each group consisted of four to six male littermates. At the end of the experiment the animals were sacrificed, and their plasma and bile were collected and gross pathology and histology were performed.

For histological processing the tissue specimens were fixed in 10% buffered formalin, dehydrated, and paraffin-embedded. Sections 2- to 3-μm thick were cut with a microtome (Leica RM2155, Germany). The sections were deparaffinized and rehydrated before hematoxylin/eosin and van Gieson's elastica stainings and periodic acid-Schiff reaction for examination in a light microscope (Olympus, Tokyo, Japan). Van Gieson's elastica was used for combined staining of elastic substrates and connective tissue. After rehydration, the sections were stained for 15

min in 0.5% resorcine/fuchsin solution in 70% ethanol. Thereafter, the sections were rinsed in distilled water, differentiated in 96% ethanol, and transferred to Weigert's iron-hematoxylin (nuclear staining) for 10 min. The sections were rinsed in distilled water, differentiated in HCl/ethanol, and rinsed in tap water for 30 min. In a third step the sections were stained in a picric acid/thiazine red mixture (10:0.2) for 10 min, rinsed in distilled water containing picric acid, and dehydrated and coverslipped by an automated coverslipper (Sakura). Periodic acid-Schiff reaction was used to demonstrate poly- and mucopolysaccharides and muco- and glycoproteins. After rehydration the sections were put into 0.5% periodic acid for 5 min at room temperature, rinsed in distilled water, and transferred to fuchsin/sulfurous acid (15 min at room temperature). The sections were washed three times in $\bar{}_2$ water, tap water (5 min), counterstained with hemalaun, rinsed in tap water, dehydrated, and coverslipped as described above. Heart and aorta were processed separately for lipid staining with Sudan III. The heart was taken out, fixed in 10% buffered formalin for at least 48 h, incubated at 37 °C in 5% gelatin for 2.5 h, in 10% gelatin for 2.5 h and in 20% gelatin overnight. The hearts were put into a gelatin block and frozen in isopentane (−35 °C) for further processing in a cryostat (Leica, Frigocut). In brief, the hearts were cut in 10-μm thickness until the 3-valve cusps at the junction of the aorta to the heart and an aorta that was round in shape was seen. Several sections were taken at this level, stained with Sudan III, and counterstained with hemalaun. The sections were examined under a light microscope. The whole aorta was fixed in 10% buffered formalin, rinsed in tap water, cut open, blocked with needles, and stained with Sudan III. The aortas were examined for atherosclerotic plaques. A total of six animals were examined by Pathology Associates Int. (Frederick, MD, project no. 3116-101 and 97-111-09), and 18 mice were analyzed at the Max-Delbrück-Center (Berlin, Germany).

Vitamin Analysis—Vitamin A and vitamin E levels were analyzed by high pressure liquid chromatography as described (35). 25-Hydroxyvitamin D was analyzed by a radioimmunoassay (36) using a kit from Nichols Institute Diagnostics. The accuracy of this method has been ascertained by a method based on isotope dilution-mass spectrometry (37). 1,25-Dihydroxyvitamin D was analyzed with a radioreceptor method (38) using a kit from Incstar (Incstar Corp., Stillwater, MN).

Lipid and Steroid Analysis—Cholesterol and triglycerides in serum were analyzed with standard enzymatic photometric methods with use of commercial kits (Boehringer Mannheim, Germany). Cholesterol in feces was analyzed by isotope dilution-mass spectrometry with $^2H_6$-labeled cholesterol as internal standard (39). Cholesterol levels in tissue extract were analyzed by the same method. The tissues were frozen in liquid nitrogen, pulverized mechanically, and extracted with chloroform/methanol (2:1, v/v). In both cases, a hydrolysis step was included, and thus the method detects both free and esterified cholesterol. Oxysterols (7α-hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol in serum and in various organs) were analyzed by isotope dilution-mass spectrometry with use of deuterium-labeled internal standards as described previously (40). The procedure includes a saponification step and thus the method includes both free and esterified steroid. The interassay variation in this assay is below 5% (40).

Bile Acid Analysis—Hydrolyzed bile acids in bile and in feces were analyzed by combined gas chromatography under the conditions described previously (41) using a Hewlett-Packard 5970 mass specific detection instrument equipped with a 0.33-μm phase HP-ultra 1 column. All samples were hydrolyzed prior to extraction, methylated with diazomethane, and trimethylsialylated prior to analysis (41). The samples were analyzed by the repetitive scanning method for identification of all bile acids present in the samples (*cf.* Fig. 5). In addition cholic acid, chenodeoxycholic acid, deoxycholic acid, and lithocholic acid were quantitated by isotope dilution-mass spectrometry with use of deuterium-labeled internal standards and selected ion monitoring as described previously (41). The other bile acids were quantitated from the chromatogram (total ion current) obtained in the analysis of material to which no internal deuterated standards had been added (Fig. 5). The peak area of the trihydroxy bile acids was compared with the peak area of cholic acid. The amount of cholic acid had been quantitated by isotope dilution-mass spectrometry in a separate analysis as described above. The peak area of the dihydroxy bile acids was compared with the peak area of deoxycholic acid. Deoxycholic acid had been quantitated by isotope dilution-mass spectrometry in a separate experiment. In a few cases the gas chromatographic peak was found to contain contaminating interfering compounds (this was the case in the assay of the small amounts of some muricholic acid isomers in the material from the *cyp27*$^{-/-}$ mice). In these cases a specific ion at m/z 195, specific for 6-hydroxylated bile acids, was used for assay.

Unhydrolyzed bile acids in bile and urine were analyzed by electrospray mass spectrometry using a Quattro triple quadropol mass spectrometer (Fig. 4). The general conditions used for pretreatment and analysis were similar to those used for analysis of bile acids by fast atom bombardment-mass spectrometry (42) The samples (a few μl of bile and 40–300 μl of urine) were diluted with water to a 1-ml volume and applied to Sep-Pak C18 columns (Waters, Milford, MA). The columns were washed with water (5–10 ml) and eluted with methanol (2 ml). After evaporation of the solvent, the samples were dissolved in acetonitrile/distilled water (1:1, v:v). Of this solution 10 μl was introduced into the mass spectrometer via loop injection with acetonitrile/water as mobile phase.

Assay of Lathosterol—This assay was performed with isotope dilution-mass spectrometry and utilization of deuterium labeled lathosterol as internal standard as described previously (43).

Experiments with 7β-^{3}H-Labeled 7α-Hydroxycholesterol—7β-^{3}H-labeled 7α-hydroxycholesterol with a specific radioactivity of 70×10^6 cpm/mg was synthesized as described previously (44). The steroid, about 9×10^6 cpm, was dissolved in 0.2 ml of ethanol, and this solution was added to 1 ml of 0.9% NaCl (w/v) containing 1% bovine serum albumin (w/v). The solution was injected intraperitoneally in one *cyp27*$^{+/+}$ and one *cyp27*$^{-/-}$ mouse. Feces was collected during three 24-h periods after this injection. The different fecal portions were refluxed with ethanol for 24 h. The ethanol was evaporated, and the material was hydrolyzed with KOH in aqueous ethanol (41). The hydrolyzed alkaline material was diluted with water and extracted three times with hexane. After acidification with diluted HCl, the water phase was extracted three times with diethyl ether. Radioactivity was measured in aliquots of the hexane phase containing neutral steroids and of the ether phase containing bile acids.

RESULTS

Cloning of the Mouse cyp27 and Construction of the Targeting Plasmid—Southern blot analysis on genomic DNA extracted from SV129 female mice resulted in a partial restriction map of the mouse *cyp27* and revealed the presence of a single copy gene (data not shown). We disrupted the *cyp27* gene by inserting the *neo* gene and deleting 71-bp *Bam*HI fragment from exon 8 (Fig. 1*b*) which is adjacent to the putative heme-binding site. Electroporation of the targeting vector to ES cells resulted in colonies from which extracted DNA revealed a ~5-kb fragment that corresponded to the targeted allele by Southern blot analysis (Fig. 2*a*). These cells were used to establish *cyp27* knock-out mice (Figs. 1*c* and 2*a*). Northern blot analysis of RNA extracted from wild type and mutant mice revealed a 1.9–2.4-kb band in the normal mice and 3.9-kb band in the homozygote *cyp27*$^{-/-}$ animals (Fig. 2*b*). Immunoblot analysis revealed no immunoprecipitable protein in the homozygote mice (Fig. 2*c*). Mutant mice were generated and genotyped by using three independent PCR reactions (Fig. 3).

Pathological Analysis—At the age of 3 weeks, the mice were weaned, and at the age of 4 weeks littermates in groups of 4–6 animals were allocated to the different cages. A total of 18 animals were used for these experiments and were fed a normal chow diet for 3 or 6 months. In general, all animals gained weight and behaved normally. No gross abnormalities were detected. Also a detailed histological analysis of all major organs and the vascular system revealed no abnormal findings. Diffuse hepatocytic cytoplasmic pallor grade 2–3 was observed, which is a normal finding in well-nourished animals.

Formation of Bile Acids in cyp27$^{-/-}$ *Mice*—With the use of combined gas chromatography-mass spectrometry as described under "Experimental Procedures," the concentration of bile acids in a sample of hydrolyzed bile from a *cyp27*$^{-/-}$ mouse was found to be only about 0.9 mg/ml as compared with about 8 mg/ml in a sample from a *cyp27*$^{+/+}$ mouse. As shown in Table I, cholic acid was the dominating bile acid, and the amount of trihydroxy bile acids was almost 20-fold higher than the amount of dihydroxy bile acids in both bile samples.

The alkaline hydrolysis used in the above analysis does not cleave sulfate esters and does not give information about the

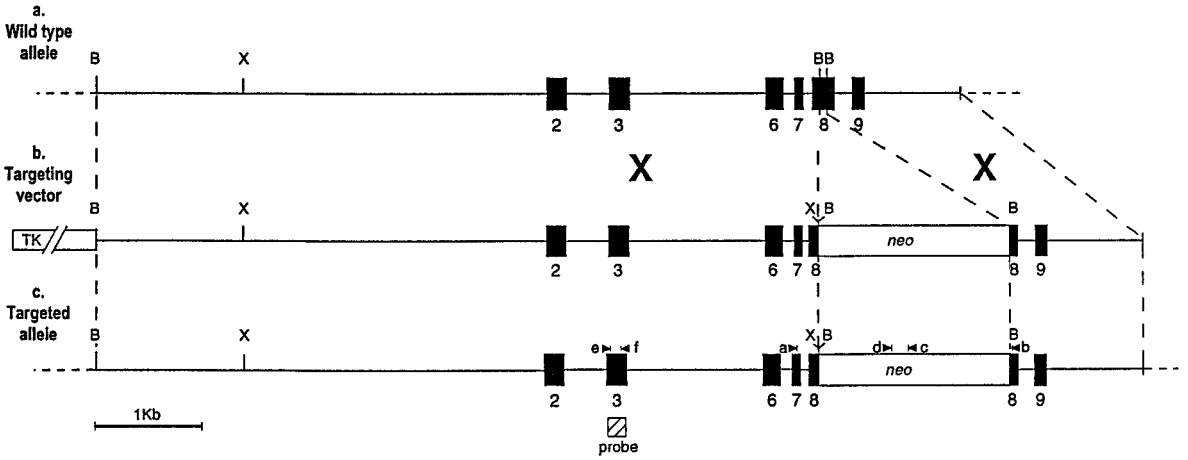

FIG. 1. **Construction of the targeting plasmid.** Schematic representation of exons 2–9, and the location of the restriction sites for *Bam*HI (*B*), *Xba*I (*X*), as well as the neomycin (*neo*) and the thymidine kinase (*TK*) sequences are shown. The probe used for screening the library and Southern blot analysis is shown (*probe*). Oligonucleotides used for PCR-based genotyping (*a–d*) and oligonucleotides used for preparing the probe (*e* and *f*) are also shown.

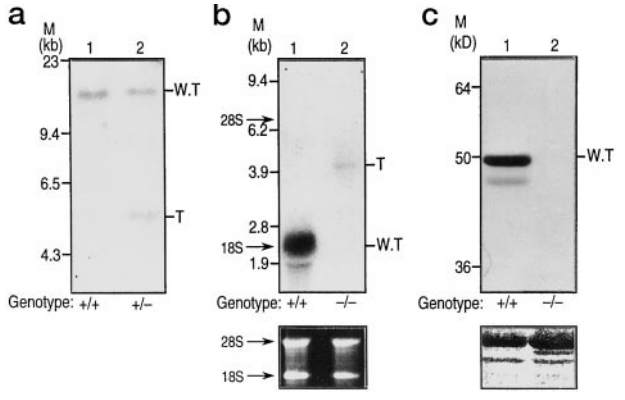

FIG. 2. **Verification of the *cyp27* disruption.** *a*, Southern blot analysis of DNA obtained from ES cells of non-targeted cells (*1*) and targeted cells (*2*). *M*, molecular weight marker, bacteriophage λ DNA digested with *Hin*dIII restriction endonuclease. *b*, blot analysis of RNA obtained from livers of control (*1*) and homozygote (*2*) mice. *M*, molecular weight marker, Kodak IB 76200 (Eastman Kodak), the location of the migration of ribosomal RNA is shown. Quantification (1 μg/lane) is verified using ethidium bromide staining (*lower panel*). *c*, immunoblotting of total liver mitochondrial protein obtained from control mouse (*1*) and from a homozygote mouse (*2*) with polyclonal antibody is shown. The *lower panel* represents Ponceau (Sigma) staining of the filter used for hybridization and reveals the presence of similar quantities of protein in both lanes.

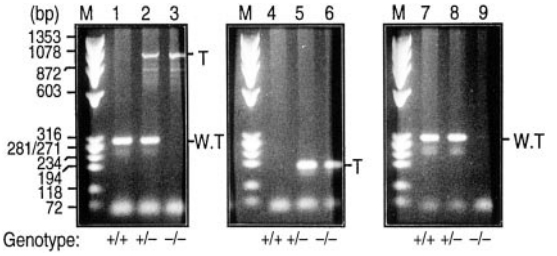

FIG. 3. **PCR-based genotyping.** *Lanes 1–3* represent the results of a PCR reaction that included oligonucleotides a, b, and c (Fig. 1*c*). Oligonucleotides a and b amplify the normal allele and a and c the targeted allele. *Lanes 4–6* represent the results of PCR amplification of the targeted allele only, using oligonucleotides c and d (Fig. 1*c*, *neo* sequences). *Lanes 7–9* represent the results of PCR amplification of the wild type allele only, using oligonucleotides a and b (Fig. 1*c*). *M*, molecular weight marker; ϕ X, *Hae*III-digested DNA. The results for the wild type (*WT*) and targeted (*T*) alleles are shown.

type of conjugates present in the bile. In order to get qualitative information about the type of conjugates, samples of unhydrolyzed bile from a $cyp27^{+/+}$ and a $cyp27^{-/-}$ mouse were also analyzed by electrospray mass spectrometry. As shown in Fig. 4, the major peak both in the analysis of the $cyp27^{+/+}$ and in the analysis of the $cyp27^{-/-}$ appeared with a m/z at 514, corresponding to taurine conjugate of trihydroxy bile acids. In addition, there was a minor peak at m/z 498, corresponding to taurine conjugate of dihydroxy bile acids. In the analysis of the bile sample from the $cyp27^{-/-}$ mouse, small peaks also appeared at m/z 531 and m/z 545. Most likely, these peaks correspond to sulfate ester of dihydroxy bile acid and monodihydroxy bile acid. Interestingly, trace amounts of compounds with m/z 611, 627, and 643 were present in the bile sample from the $cyp27^{-/-}$ mouse. These peaks correspond to quasimolecular ions of glucuronides of bile alcohols containing 27-carbon atoms and four, five, and six hydroxyl groups (42). No such compounds could be detected in the analysis of the bile sample from the $cyp27^{+/+}$ mouse.

That the production of bile acids was markedly decreased in the $cyp27^{-/-}$ mice was also confirmed by combined gas chromatography-mass spectrometry of feces obtained from four $cyp27^{+/+}$ and four $cyp27^{-/-}$ mice (Fig. 5 and Table I). As shown in Table I, the excretion of bile acids in feces was about 78 μg/g in the $cyp27^{-/-}$ mouse and about 430 μg/g in the $cyp27^{+/+}$ mouse. The composition of the fecal bile acids was similar in the two groups, although the relative amount of dihydroxy bile acids was somewhat higher in the $cyp27^{-/-}$ group (about 50%) as compared with the $cyp27^{+/+}$ group (about 30%).

Bile acids in feces were also analyzed in a $cyp27^{+/-}$ mouse. The amount of bile acids (about 400 μg/g feces) as well as the composition was very similar to those found in feces of $cyp27^{+/+}$ mice.

In order to further ascertain that there is a reduced formation of bile acids in $cyp27^{-/-}$ mice, one $cyp27^{-/-}$ mouse and one $cyp27^{+/+}$ mouse was injected with the same amount of 7β-^{3}H-labeled 7α-hydroxycholesterol. Since 7α-hydroxycholesterol is an intermediate in bile acid biosynthesis occurring after the rate-limiting step, a rapid formation of bile acids from this compound can be expected in normal mice. In accordance with this, 1.6×10^6 cpm were found in the bile acid containing ether extract of the feces collected during 72 h after the injection of 9×10^6 cpm in the $cyp27^{+/+}$ mouse. The hexane extract containing neutral steroids contained 0.4×10^6 cpm. Only 0.2×10^6 cpm were found in the bile acid containing extract from the $cyp27^{-/-}$ mouse and 1.0×10^6 cpm in the extract containing neutral steroids. The formation of radioactive bile acids from 7β-^{3}H-labeled 7α-hydroxycholesterol in the $cyp27^{-/-}$ mouse

TABLE I
Analysis of fecal and biliary bile acids in wild type and cyp27$^{-/-}$ mice

Bile acid	Fecal bile acids		Biliary bile acids	
	+/+	−/−	+/+	−/−
	μg/g feces[a]		*mg/ml bile*[b]	
3α-Hydroxy-5β-cholanoic acid (lithocholic acid) (I)	20 ± 5	2 ± 1	<0.01	<0.01
Unknown 3,12-Dihydroxycholanoic acid (II)	22 ± 3	5 ± 2	n.d.	n.d.
3α,12α-Dihydroxy-5β-cholanoic acid (deoxycholic acid) (III)	91 ± 11	26 ± 8	0.29	0.02
3α,6β,7α-Trihydroxy-5β-cholanoic acid (α-muricholic acid) (IV)	44 ± 14	10 ± 6	0.82	0.02
3α,7α-Dihydroxy-5β-cholanoic acid (chenodeoxycholic acid) (V)	4 ± 1	0.9 ± 0.1	0.19	0.03
3α,7α,12α-Trihydroxy-5β-cholanoic acid (cholic acid) (VI)	27 ± 4	14 ± 1	5.67	0.70
3,12-Dihydroxycholanoic acid (VII)	18 ± 6	7 ± 3	n.d.	n.d.
3α,6β,7β-Trihydroxy-5β-cholanoic acid (β-muricholic acid) (VIII)	63 ± 9	0.8 ± 0.8	0.60	0.05
3α,6α,7β-Trihydroxy-5β-cholanoic acid (ω-muricholic acid) (IX)	112 ± 18	10 ± 2	0.22	0.05
Unknown 6-hydroxylated trihydroxycholanoic acid (X)	29 ± 4	4 ± 2	n.d.	n.d.
	430 ± 55	78 ± 18[c]	7.79	0.87

[a] Mean ± S.E., $n = 4$.
[b] Results are given from analysis of bile from one animal only from each group. Results obtained from additional analysis of bile from *cyp27*$^{+/+}$ and *cyp27*$^{-/-}$ mice were similar.
[c] $p < 0.001$, Student's t test.

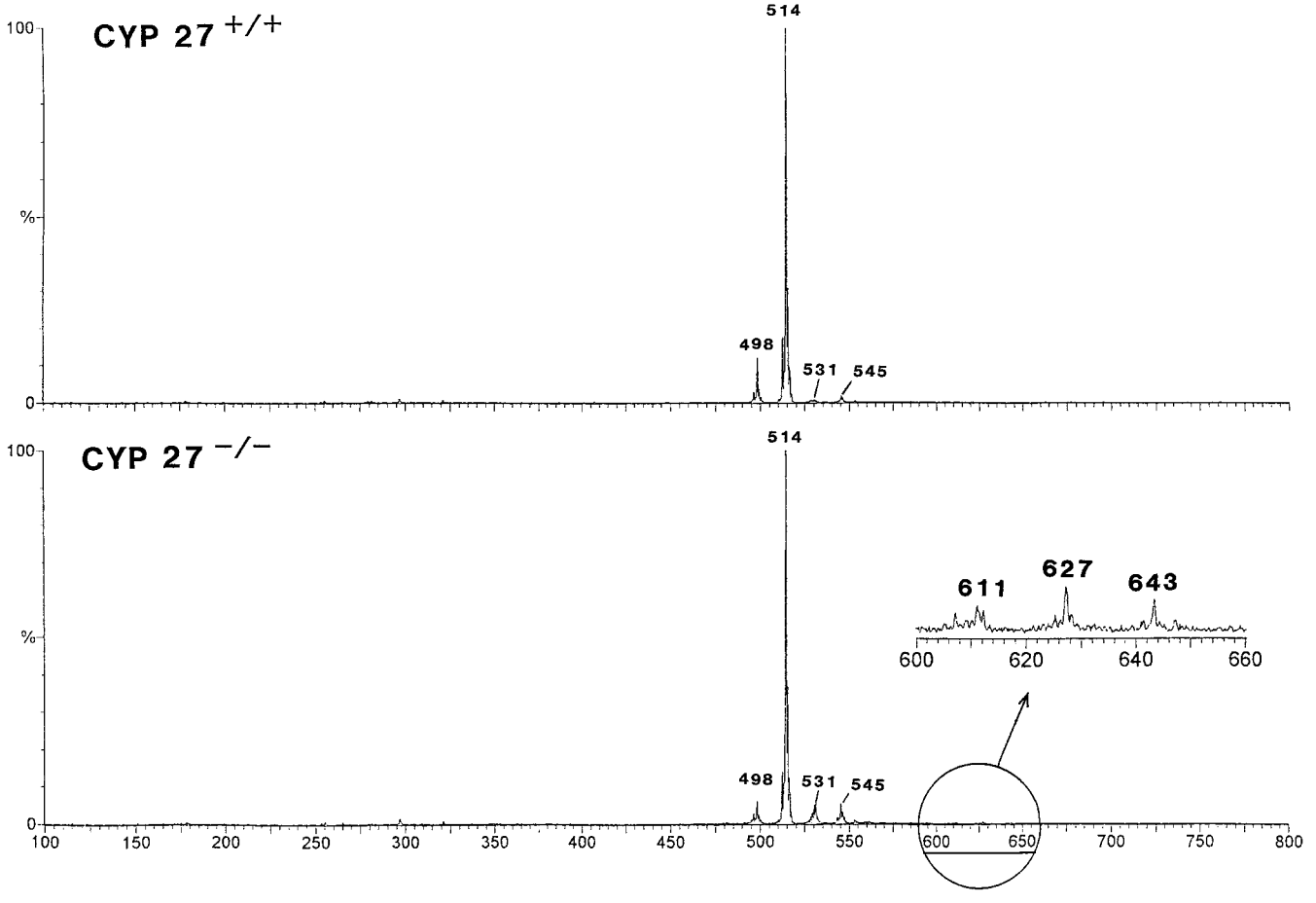

FIG. 4. **Electrospray mass spectrometry of unhydrolyzed bile from a *cyp27*$^{+/+}$ and a *cyp27*$^{-/-}$ mouse.** For experimental details, see "Experimental Procedures."

was thus only about 13% of that in the *cyp27*$^{+/+}$ mouse. It should be pointed out, however, that due to the expanded pool of 7α-hydroxycholesterol in the *cyp27*$^{-/-}$ mice (Table III), the conversion of the trace amount of labeled intermediate may not be an adequate reflection of the conversion of the endogenous compound.

The possibility that bile acids were excreted in urine was also tested with electrospray mass spectrometry of un-hydrolyzed urine samples from *cyp27*$^{+/+}$ and *cyp27*$^{-/-}$ mice. No significant amounts of bile acids were seen. Small peaks at m/z 627, 643, and 657 were seen in the analysis of urine from *cyp27*$^{-/-}$ mice but not from *cyp27*$^{+/+}$ mice. The latter peaks are likely to be due to glucuronides of bile alcohols containing 27 carbon atoms and five, six, and seven hydroxyl groups (42) (*cf.* above).

The low production of bile acids is likely to result in an up-regulation of the cholesterol 7α-hydroxylase due to a reduced negative feedback suppression of this enzyme. In accordance with this, the concentration of 7α-hydroxycholesterol in the liver, the kidney, and the brain was markedly higher in the *cyp27*$^{-/-}$ mice than in the *cyp27*$^{+/-}$ and in the *cyp27*$^{+/+}$ mice (Table II). It is well established that circulating levels of 7α-hydroxycholesterol reflect the activity of the cholesterol 7α-

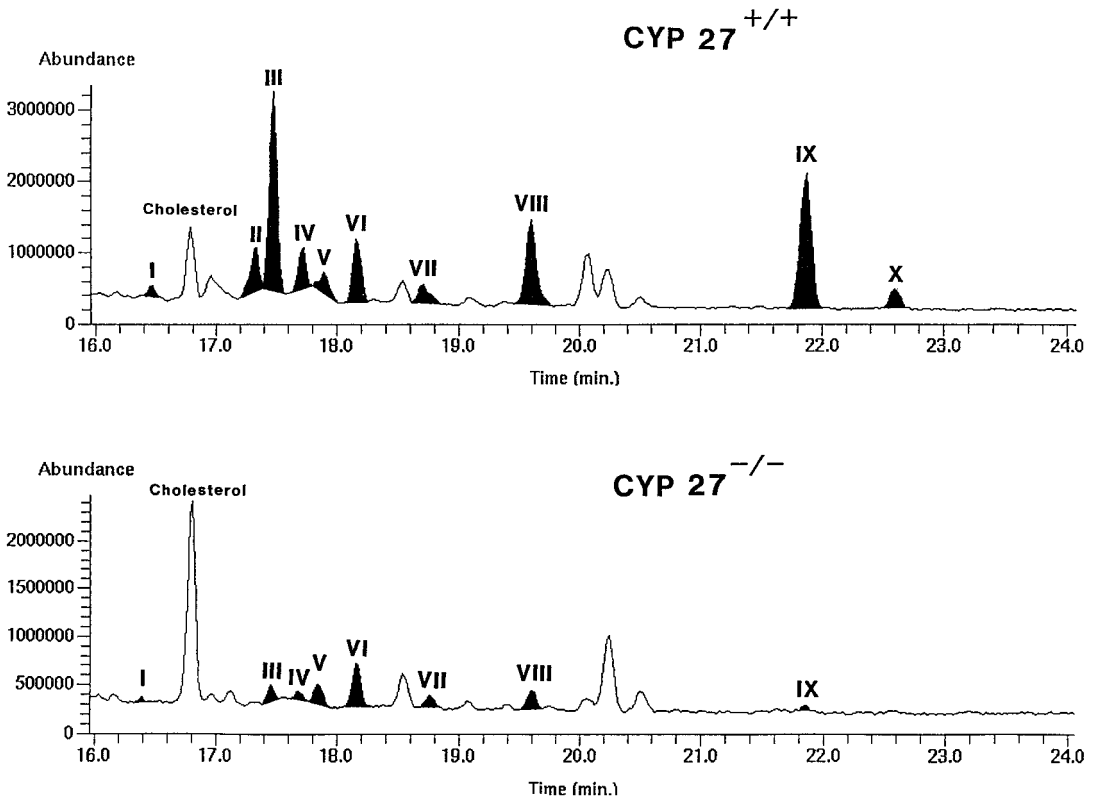

FIG. 5. **Gas chromatogram obtained in a gas chromatography-mass spectrometry analysis of methylated and trimethylsilyl ether-derivatized hydrolyzed extract of feces from a control mouse (*cyp27*$^{+/+}$) and a sterol 27-hydroxylase-deficient mouse (*cyp27*$^{-/-}$).** An equal amount of a daily excretion of feces was analyzed. *I*, lithocholic acid (3α-hydroxy-5β-cholanoic acid); *II*, isomer of a 3,12-dihydroxy-5β-cholanoic acid; *III*, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); *IV*, α-muricholic acid (3α,6β,7α-trihydroxy 5β-cholanoic acid); *V*, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); *VI*, cholic acid, (3α,7α,12α-trihydroxy-5β-cholanoic acid); *VII*, isomer of a 3,12-dihydroxycholanoic acid; *VIII*, β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid; *IX*, ω-muricholic acid (3α,6α,7β-trihydroxy-5β-cholanoic acid); *X*, *allo*-ω-muricholic acid, (3α,6α,7β-trihydroxy-5α-cholanoic acid).

TABLE II
Content of 7α-hydroxycholesterol in different organs from wild type, cyp27$^{+/-}$ *and cyp27*$^{-/-}$ *mice*

Organ	*cyp27* genotype		
	+/+	+/−	−/−
	ng/100 mg tissue		
Liver	70	70	880
Kidney	62	63	377
Brain	54	28	249

TABLE III
Serum levels of some oxysterols in cyp$^{+/+}$, *cyp27*$^{+/-}$, *and cyp27*$^{-/-}$ *mice*

Mean ± S.E., $n = 5$ (+/+ and +/−) and 6 (−/−).

Oxysterol	*cyp27* genotype		
	+/+	+/−	−/−
	μg/ml		
7α-Hydroxycholesterol	0.47 ± 0.20	0.40 ± 0.16	2.2 ± 1.0
24-Hydroxycholesterol	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.03
27-Hydroxycholesterol	0.08 ± 0.01	0.04 ± 0.00	<0.001

hydroxylase in the liver (45). The concentration of circulating 7α-hydroxycholesterol was found to be 4–10-fold higher in the *cyp27*$^{-/-}$ mice than in the two other genotypes (Table III).

That the cholesterol 7α-hydroxylase was up-regulated was also confirmed by RNA blot analysis (data not shown). Thus the ratio between the *cyp7* and the actin mRNA was 2.7 ± 0.5 (n = 7) and 0.3 ± 0.1 (n = 6), in the livers of the *cyp27*$^{-/-}$ and the *cyp27*$^{+/+}$ mice, respectively.

Cholesterol Synthesis—The reduced synthesis of bile acids in the knock-out mice leads to reduced absorption of cholesterol, which may lead to a compensatory increased synthesis. The up-regulation of the cholesterol 7α-hydroxylase can also be expected to lead to a secondary up-regulation of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase (46–48). In accordance with this, the hepatic levels of HMG-CoA reductase mRNA was found to be 2–3-fold higher in the knock-out mice than in the wild type (data not shown).

It is well documented that up-regulation of HMG-CoA reductase in the liver leads to increased levels of the precursor lathosterol in the circulation (49). The level of lathosterol in the circulation of three *cyp27*$^{-/-}$ mice was found to be 769 ± 56 ng/ml (mean ± S.E.) as compared with 231 ± 90 ng/ml in three *cyp27*$^{+/+}$ mice ($p < 0.01$, Student's t test).

Circulating Levels of Fat-soluble Vitamins—As shown in Table IV, the levels of vitamin A were about the same in the wild type and in the *cyp27*$^{-/-}$ mice. Vitamin E levels in the *cyp27*$^{-/-}$ mice were somewhat lower than those of the wild type, but this difference was not significant from a statistical point of view. Levels of 25-hydroxyvitamin D were clearly higher in the *cyp27*$^{-/-}$ mice than in the wild type ($p < 0.01$, Student's t test). Levels of 1,25-dihydroxyvitamin D were similar in the two groups of mice.

Circulating Levels of Cholesterol and Triglycerides, Fecal Excretion of Cholesterol—The circulating levels of cholesterol (total cholesterol) varied between 1.5 and 3.0 mmol/liter in both the wild type and the *cyp27*$^{-/-}$ mice on a normal diet. The triglyceride levels in the three groups of mice varied between 0.2 and 0.8 mmol/liter.

The very low production of bile acids in the *cyp27*$^{-/-}$ mice could be expected to result in increased fecal excretion of fat in feces. The excretion of cholesterol was 3.4 ± 0.3 (n = 5) and

TABLE IV
Levels of fat-soluble vitamins or vitamin metabolites in the circulation of wild type and cyp27$^{-/-}$ mice

Mean ± S.E., $n = 3$.

Vitamin	*cyp27* genotype	
	+/+	−/−
Vitamin A (μmol/liter)	1.2 ± 0.1	1.3 ± 0.3
Vitamin E (μmol/liter)	9.7 ± 2.0	6.0 ± 1.9
25-Hydroxyvitamin D_3 (μg/liter)	42 ± 7	106 ± 9
1,25-Dihydroxyvitamin D_3 (μg/liter)	0.13 ± 0.04	0.14 ± 0.03

1.9 ± 0.2 mg/g ($n = 5$) feces in the *cyp27*$^{-/-}$ and the *cyp27*$^{+/+}$ mice, respectively ($p = 0.05$).

Levels of Cholestanol in Liver and Circulation—The ratio between cholestanol and cholesterol was found to be 0.009 ± 0.003, 0.011 ± 0.003, and 0.016 ± 0.006 in livers of the *cyp27*$^{-/-}$, the *cyp27*$^{+/-}$, and the *cyp27*$^{+/+}$ mice, respectively. Similar ratios were found in the circulation (not shown).

DISCUSSION

Consequences of the Lack of Sterol 27-Hydroxylase for Bile Acid Synthesis—It is evident that a lack of the sterol 27-hydroxylase has a more dramatic effect on production of bile acids in mice than in humans. In patients with CTX the enzyme deficiency leads to a markedly reduced production of chenodeoxycholic acid, whereas production of cholic acid is normal or almost normal (10). The latter production is most probably due to a microsomal 25-hydroxylase, active on 5β-cholestane-3α,7α,12α-triol. The product 5β-cholestane-3α,7α,12α,25-tetrol can be converted into cholic acid in a pathway involving cleavage of acetone from the steroid side chain (*cf.* above). The microsomal 25-hydroxylase is considerably more active in human liver microsomes than in rat liver microsomes (51). In preliminary experiments the activity of this enzyme was found to be about equal in rat and mouse liver. The relatively low activity of the microsomal 25-hydroxylase in mouse liver may thus explain why *cyp27*$^{-/-}$ mice are unable to compensate for the lack of the sterol 27-hydroxylase by formation of bile acids through the alternative 25-hydroxylase mechanism.

The small amounts of bile acids found in the *cyp27*$^{-/-}$ mice might in part have been formed by the sterol 25-hydroxylase pathway. Small amounts of bile alcohols were found in bile and urine of *cyp27*$^{-/-}$ mice, and trace amounts of 5β-cholestane-3α,7α,12α,25-tetrol could in fact be identified in feces of one of the *cyp27*$^{-/-}$ mice.

In contrast to humans, rat liver contains a microsomal 26-hydroxylase that is able to catalyze 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (52). The product, 5β-cholestane-3α,7α,12α,26-tetrol, being an isomer to 5β-cholestane-3α,7α,12α,27-tetrol, might well be further converted into cholic acid. Whether or not mouse liver microsomes contain such a 26-hydroxylase is not known with certainty. If such enzyme activity exists, it may also explain part of the small amounts of bile acids formed in the *cyp27*$^{-/-}$ mice. At the present state of knowledge, we also cannot exclude that a sterol 24-hydroxylase present in the brain (53) may be of some importance for the conversion of cholesterol into bile acids in the *cyp27*$^{-/-}$ mice.

In accordance with the findings in patients with CTX, the lack of the sterol 27-hydroxylase resulted in a compensatory up-regulation of the cholesterol 7α-hydroxylase in the *cyp27*$^{-/-}$ mice. The circulating levels of 7α-hydroxycholesterol were thus markedly elevated, and RNA blot analysis showed about 9-fold increased concentrations of cholesterol 7α-hydroxylase mRNA in the liver.

Formation of Cholestanol in cyp27$^{-/-}$ *Mice*—One of the characteristics of CTX is an increased production of cholestanol (10). This production is most probably linked to the accumulation of 7α-hydroxylated intermediates in bile acid biosynthesis, in particular 7α-hydroxy-4-cholesten-3-one. Conversion of 7α-hydroxy-4-cholesten-3-one into cholestanol requires a critical dehydration of this steroid by a microsomal dehydratase (54). We have shown that the activity of this enzyme is severalfold higher in human liver than in rat liver (54). In preliminary experiments we showed that the activity of this dehydratase is about similar in rat and in mouse liver. The relatively low activity of this enzyme may thus explain why the *cyp27*$^{-/-}$ mice did not have higher levels of cholestanol than the *cyp27*$^{+/+}$ mice.

Consequences of the Lack of Sterol 27-Hydroxylase for Transport of Cholesterol from Peripheral Tissues—Patients with CTX are known to develop premature atherosclerosis despite normal circulating levels of cholesterol (10). It has been suggested that this could be a consequence of the lack of the sterol 27-hydroxylase in macrophages in these patients (1–3). The sterol 27-hydroxylase is thus able to eliminate cholesterol from macrophages by converting it into 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid that are easily excreted from the cells. The general importance of this mechanism is reflected in the very high levels of sterol 27-hydroxylase in human macrophages (1, 2) and in the high flux of 27-oxygenated C_{27} steroids from extrahepatic tissues to the liver in humans (3). The latter flux is likely to be of similar magnitude in mice and in humans, as judged from the relatively high levels of circulating 27-hydroxycholesterol (Table III). Under the conditions employed, however, the *cyp27*$^{-/-}$ mice did not produce visible xanthomas or atheromas. At the present state of knowledge, it cannot be excluded that the accumulation of cholestanol and/or C_{27} bile alcohols that are seen in CTX patients but not in the *cyp27*$^{-/-}$ mice is the most critical factor for the development of xanthomas and atheromas in CTX patients.

Consequences of the Lack of the Sterol 27-Hydroxylase for Cholesterol Synthesis and Homeostasis—27-Hydroxycholesterol is known to be a potent down-regulator of HMG-CoA reductase in cultured cells (7, 8). The importance of this mechanism is, however, controversial. If the sterol 27-hydroxylase has a key role in cholesterol homeostasis, a lack of the enzyme would be expected to be associated with a markedly increased synthesis of cholesterol and elevated circulating levels of cholesterol in the circulation. CTX patients are, however, known to have normal circulating levels of cholesterol, and this was found to be the case also in the present *cyp27*$^{-/-}$ mice. Patients with CTX are known to have an overall increased cholesterol synthesis, most probably secondary to the up-regulated cholesterol 7α-hydroxylase (10). That the increased cholesterol synthesis in the present sterol 27-hydroxylase-deficient mice is secondary to the up-regulated cholesterol 7α-hydroxylase seems likely in view of the finding that the synthesis of the latter enzyme was increased much more than the synthesis of HMG-CoA reductase. The up-regulated hepatic HMG-CoA reductase may be a compensation for a consumption of cholesterol due to the highly up-regulated cholesterol 7α-hydroxylase (*cf.* Ref. 48).

Consequences of the Lack of the Sterol 27-Hydroxylase for Metabolism of Vitamin D—Sterol 27-hydroxylase is able to 25-hydroxylate vitamin D (4). Since the liver mitochondrial fraction of a patient with CTX has been found to possess some 25-hydroxylase activity toward vitamin D (55) and since the microsomal fraction of liver homogenate also contains this activity (6), the relative importance of the sterol 27-hydroxylase for this reaction is difficult to evaluate. Reduced bone density and reduced circulating levels of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D have also been reported in some

CTX patients (23). Other patients have normal levels of 25-hydroxyvitamin D, however (10).[2] A serum pool from three *cyp27*$^{-/-}$ mice had somewhat higher concentrations of 25-hydroxyvitamin D than a serum pool from three *cyp27*$^{+/+}$ mice. In view of this, sterol 27-hydroxylase seems to be of little importance for 25-hydroxylation of vitamin D in mice. Recently it was shown that sterol 27-hydroxylase has some 1α-hydroxylase activity toward 25-hydroxyvitamin D_3 (5). The circulating concentration of 25-dihydroxyvitamin D was, however, somewhat higher in *cyp27*$^{-/-}$ mice than in *cyp27*$^{+/+}$ mice, whereas the corresponding concentrations of 1,25-dihydroxyvitamin D were similar in the two groups of mice. Thus it seems less likely that sterol 27-hydroxylase is of importance in formation of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in this species.

Consequences of the Reduced Formation of Bile Acids in cyp27$^{-/-}$ *Mice*—Surprisingly, the markedly reduced production of bile acids in the *cyp27*$^{-/-}$ mice was not associated with any symptoms of malabsorption. The content of cholesterol and fatty acids in feces was thus only moderately increased, and the circulating levels of the fat-soluble vitamins A and E were normal or only slightly reduced. It is interesting to compare this situation with the situation in mice with a disruption of the *cyp7* (56). During the first 4 weeks of life, these mice had a severe fat malabsorption with very low circulating levels of 25-hydroxyvitamin D_3 and vitamin E and high content of lipids in stool. After this period a normalization occurred, most probably due to induction of an oxysterol 7α-hydroxylase in the liver. The adult *cyp27*$^{-/-}$ mice had an excretion of bile acids in stool that was reduced by about 80% as compared with the wild type. Evidently, this low degree of production of bile acids is sufficient to prevent fat malabsorption.

Is the Cyp 27$^{-/-}$ *Mouse Suitable as an Animal Model for CTX?*—It is evident that a disruption of the *cyp27*$^{-/-}$ gene in a mouse on a normal diet does not lead to the metabolic, neurologic, and vascular disturbances found in patients with a corresponding genetic defect. The compensatory mechanism in humans (activation of the 25-hydroxylase pathway) and the consequences of accumulation of bile acid intermediates (accumulation of cholestanol) were not observed in the *cyp27*$^{-/-}$ mice. It is possible that the enzymatic defect in the *cyp27*$^{-/-}$ mice may be more important when increasing the dietary intake of cholesterol. In preliminary experiments an increased death rate was observed for *cyp27*$^{-/-}$ mice when fed an atherogenic diet. No explanation for this finding has been obtained thus far, however. Experiments with the goal of clarifying the importance of the sterol 27-hydroxylase in mice with additional genetic defects and under different dietary conditions are now in progress.

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